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Dynamics of circulating TNF during adalimumab treatment using a drug-tolerant TNF assay

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Overline: Autoimmunity

One sentence summary: TNF concentrations stabilize in patients with rheumatoid arthritis during longterm adalimumab therapy and are associated with anti-drug antibodies.

Abstract

Patients with rheumatoid arthritis (RA) can be successfully treated with tumor necrosis factor- (TNF) inhibitors, including the monoclonal antibody adalimumab. Once in remission, a proportion of patients can successfully discontinue treatment, indicating that blocking TNF is no longer required for disease control. To explore the dynamics of circulating TNF during adalimumab treatment, we developed a competition enzyme-linked immunosorbent assay that can quantify TNF in the presence of large amounts of TNF-inhibitor, i.e. a 'drug-tolerant' assay. In 193 consecutive adalimumab-treated patients with RA we demonstrated that circulating TNF increased on average >50-fold upon treatment and reached a stable concentration in time in the majority of patients. A similar increase in TNF was found in 30 healthy volunteers after one dose of adalimumab. This implies that TNF in circulation during anti-TNF treatment is not primarily associated with disease activity. During treatment, TNF was in complex with adalimumab, and could be recovered as inactive 3:1 adalimumab:TNF complexes. No quantitative association was found between TNF and adalimumab concentrations. Remarkably, low TNF concentrations at week four were associated with a higher frequency of anti-drug antibodies (ADAs) at subsequent time points, less frequent methotrexate use at baseline, and less frequent remission after 52 weeks. Also in healthy volunteers, early low TNF concentrations associated with ADAs. In conclusion, longitudinal TNF concentrations are mostly stable during adalimumab treatment and may therefore not predict successful treatment discontinuation. However, early low TNF is strongly associated with ADA formation and may be used as timely predictor of non-response towards adalimumab treatment.

Introduction

Biological disease-modifying antirheumatic drugs (bDMARDs) targeting tumor necrosis factor (TNF) are efficacious in the treatment of immune-mediated inflammatory diseases, such as rheumatoid arthritis

(RA), which highlights the importance of TNF as a driver of inflammation in these diseases. Quantification of TNF during anti-TNF treatment might provide insight in treatment efficacy and / or the role of TNF during treatment. Numerous studies have investigated TNF concentrations during anti-TNF treatment in patients *(1–5)*. However, the quantification of TNF is challenging for a number of reasons. First, TNF is rapidly cleared from the circulation (TNF half-life is in the order of minutes) *(6)*. Consequently, TNF concentrations in circulation are low, around the detection limit of most immunoassays, even during active disease *(1–3)*. Second, TNF is unstable in biological samples *(7, 8)*, due to continuous monomeric subunit exchange *(9)*. Finally, the trimeric TNF structure is easily compromised, e.g. during freezing and thawing of serum samples, with direct impact on TNF quantification *(10)*. It is therefore unlikely that pre-treatment TNF is a reliable biomarker.

In contrast, TNF bound to a TNF-inhibitor has a prolonged half-life, since the TNF-inhibiting antibodies themselves have a very long half-life of several weeks. This explains the observed increase in TNF concentrations shortly after initiation of anti-TNF treatment, in particular for infliximab and etanercept treatment *(1–5)*. Similar increases were found for other anti-cytokine antibodies, such as anti-interleukin (IL)-6 *(11)*. However, measurement of TNF during anti-TNF treatment is also challenging. TNF-inhibitors interfere with the quantification of TNF, likely resulting in a severe underestimation of the TNF concentration. This limitation affects all of the above mentioned studies.

Two observations suggest that TNF concentrations might be associated with clinical response. First, Charles *et al.* found that after a peak TNF concentration at day seven, after a single infliximab infusion, TNF concentrations gradually declined *(1)*. This decline was associated with a significant reduction in C-reactive protein (CRP) *(12)*. Second, it has been shown that once in remission, a proportion of patients with RA can successfully discontinue bDMARD treatment (reported range 20-79% of the patients in remission) *(13–15)*. Together, these observations suggest a mechanism where successful treatment discontinuation hinges on a decline in TNF production, thereby alleviating the need for blocking TNF.

In order to investigate the relation between TNF concentrations and clinical response, we quantified TNF serum concentrations with a drug-tolerant assay in three groups of adalimumab-treated patients with RA and in healthy volunteers, who received one dose of an adalimumab biosimilar. We expected an overall increase in circulating TNF in the first phase of treatment, followed by a decrease in TNF over two years of follow-up in patients who are in clinical remission. Following from that, monitoring TNF during anti-TNF treatment could be a potential biomarker in predicting successful treatment discontinuation.

Results

Quantification of TNF with a drug-tolerant assay

In order to quantify TNF, independent of the presence of large amounts of adalimumab during treatment, we developed a drug-tolerant competition enzyme-linked immunosorbent assay (ELISA) (Materials and Methods; Fig. 1A). A biotinylated high-affinity adalimumab mutant *(16)* was used for the detection of TNF and was selected based on its increased affinity to TNF over adalimumab (fig. S1). As a consequence of the increased affinity, the high-affinity adalimumab mutant can efficiently displace adalimumab from TNF. In the resulting assay, quantitative recovery of TNF in the presence of large amounts of TNF-inhibitor was achieved (Fig. 1B), whereas in a conventional TNF ELISA, recovery is lower than 3% (Fig. 1C). We analyzed healthy donor sera (n=70) in this drug-tolerant competition ELISA, yielding signals that would translate to a median of approximately 1.2 (range <0.9 – 6) pg/ml

TNF (fig. S2A). Based on these concentrations, the lower limit of detection was set to 5 pg/ml TNF. Furthermore, by testing sera from biologic naïve patients with RA (n=20), we found that pre-treatment TNF concentrations in patients with RA were also low, with median concentrations below 5 pg/ml TNF (fig. S2B). In contrast, in a set of anonymously collected sera from 36 adalimumab-treated patients sent in to Sanquin Diagnostic Services, we observed a wide range of TNF: 10 – 743 pg/ml (fig. S2C). Adalimumab concentrations in these samples ranged from 0.1 – 22 μg/ml (fig. S2D).

Characterization of TNF-adalimumab complexes

To investigate to what extent the TNF is free or drug-bound during adalimumab treatment, we also developed a complex ELISA (fig. S3A). In this assay, free TNF and TNF-adalimumab complexes were captured analogously to the competition ELISA, and TNF-adalimumab complexes were specifically detected with an adalimumab rabbit-anti-idiotype antibody (fig. S3B). The abovementioned sera from adalimumab-treated patients from Sanquin Diagnostic Services were also analyzed in the complex ELISA. We observed a very good correlation between the two different assays (Pearson r=0.96, $p<0.0001$; Fig. 2A), suggesting that during adalimumab treatment TNF is predominantly in complex with adalimumab.

To characterize TNF-adalimumab complexes in patients in more detail, we subsequently analyzed these complexes *ex vivo* with high-performance size-exclusion chromatography (HP-SEC). We first fractionated spike samples of free TNF and TNF-adalimumab complexes in buffer containing IVIg and albumin as carrier proteins (Fig. 2B and 2C, respectively). TNF was measured in collected fractions with the competition ELISA, shown in black. Free TNF was recovered in the fractions containing small proteins (Fig. 2B), whereas for TNF-adalimumab complexes the TNF peak shifted to the left, indicating that all TNF was in complex with adalimumab (Fig. 2C). The elution profile is consistent with a single dominant type of complex, a 3:1 adalimumab:TNF complex. Elution profiles of adalimumab:TNF complexes made by combining TNF with excess adalimumab at different ratios overlapped with each other and with those of the *ex vivo* complexes (fig. S4). This confirmed the 3:1 adalimumab-TNF

complex ratio. Next, we characterized TNF-adalimumab complexes in serum derived from patients with RA during standard-dose adalimumab treatment. A representative graph of an RA patient is shown in Fig. 2D. TNF-adalimumab complexes in patient sera (n=4) were of similar size as the *in vitro* spiked complexes. We did not observe free TNF in these patient sera.

Longitudinal TNF concentrations

Next, longitudinal TNF concentrations were quantified with the drug-tolerant competition ELISA in 193 biologic naïve patients with RA starting standard-dose adalimumab treatment (Baseline characteristics, see Table 1). Confirming our hypothesis, TNF concentrations were close to the detection limit at baseline, but strongly increased four weeks after start of adalimumab treatment (Fig. 3A). Compared to baseline, median TNF concentrations after half a year of treatment were at least 50-fold higher (median TNF of 292 pg/ml (inter quartile range (IQR) 5-2125 pg/ml at week 28). Despite the measurement of high TNF concentrations during adalimumab treatment, these high amounts of circulating TNF were complexed and thus biologically inactive in a cell viability assay, which quantifies killing of Walter and Elizabeth Health Institute (WEHI) cells by biologically active TNF (Fig. 3B). After the initial increase in the early phase of treatment, TNF concentrations remained remarkably stable over time for the majority of patients during the two-year follow-up (representative curves shown in Fig. 3C). Nevertheless, while TNF concentrations varied only slightly for individual patients, there was considerable variability in TNF concentrations between patients. However, there was only a weak overall association between TNF and adalimumab concentrations (Spearman's $\rho=0.38$, $p<0.0001$; fig. S5). In contrast, in patients in whom adalimumab became undetectable, coinciding with the appearance of anti-drug antibodies (ADAs), TNF concentrations rapidly dropped, after an initial rise from baseline to four weeks (representative curves shown in Fig. 3D).

Dose-interval prolongation and treatment discontinuation

In 21 adalimumab-treated patients with RA, who prolonged their dosing interval from every other week to once every three weeks, mean (standard deviation (SD)) adalimumab concentrations decreased from 11.0 (2.6) to 6.5 (2.2) µg/ml at week 28 (Fig. 4A). However, TNF concentrations remained completely stable in all patients; baseline median (IQR) TNF was 339 (114-825), and 383 (122-1080) pg/ml at 28 weeks after treatment prolongation. Median (IQR) change in TNF within patients from baseline to week 28 was 0 (-30.5-48) pg/ml (Fig. 4B).

Treatment discontinuation in 11 patients with RA resulted in a decrease in mean (SD) adalimumab concentration from 5.5 (2.9) to 0.6 (0.5) µg/ml at 12 weeks and 0.1 (0.1) µg/ml at 24 weeks after treatment discontinuation (Fig. 4C). TNF concentrations decreased from 381 (16-707) to 290 (2.5-755) pg/ml in the first 12 weeks and to 83 (2.5-532) pg/ml at 24 weeks after treatment discontinuation (Fig. 4D). In a minority of patients, adalimumab concentrations dropped to or below the detection limit. In those patients, TNF concentrations decreased much more rapidly. In patients in whom the adalimumab concentration was >0.1 µg/ml after 24 weeks of treatment discontinuation (n=4), TNF remained stable, suggesting that an adalimumab concentration as low as 0.1 µg/ml may be sufficient for quantitative capture of TNF *in vivo*.

Exploration of clinical response

Given the wide variation in TNF concentrations between patients during adalimumab treatment, we investigated whether this variation was related to clinical response. For these analyses, patients with an adalimumab concentration <0.1 µg/ml were excluded (week 4; n=0, week 52; n=7), as we expected that quantitative capture of TNF may be achieved with as little as 0.1 µg/ml of adalimumab in circulation. At week four, serum samples were available in 168 (87%) patients. No association was found between TNF concentrations at week four and disease activity at baseline, according to the simplified disease activity index (SDAI) (Spearman's ρ=-0.07, p=0.36). We did observe a weak inverse correlation between TNF concentrations at week four and SDAI after 52 weeks of standard-dose adalimumab treatment (Spearman's ρ=-0.21, p=0.005; Fig. 5A). Since most non-responders will drop

out before 28 weeks *(17)*, we queried whether the association between TNF concentrations and disease activity remained at later time-points. Both TNF concentration at week 52 (n=134) and during steady state (n=141), however, did not correlate with SDAI (Spearman's ρ=-0.096, p=0.27 and ρ=-0.098, p=0.25; figs. S6A and S6B, respectively). The number of visits available to determine steady state TNF concentration (two or three visits) did not affect the association.

Furthermore, we found that 43 (22%) patients developed detectable ADAs during 52 weeks of follow-up. Median TNF concentrations at week four were significantly lower in patients with detectable ADAs compared to patients without detectable ADAs (12 (IQR 5.6-90) vs 170 (70-295) pg/ml TNF, respectively, p<0.001; Fig. 5B). We validated and confirmed this relationship between TNF and SDAI and ADAs in a second, independent cohort of 193 consecutive adalimumab-treated patients with RA (baseline characteristics, see table S1**;** fig. S7). With receiver operator characteristic (ROC) analysis we found that samples with a concentration below 11 pg/ml TNF at week four gave a 51% sensitivity and 95% specificity for ADA detection after 52 weeks, with an AUC of 0.79 (Fig. 5C, black line). Inclusion of baseline methotrexate usage in the logistic regression model yielded a marginally larger AUC of 0.81 (Fig. 5C, gray line).

Importantly, only some patients (n=13) had small quantities of ADAs detectable at week four, but adalimumab concentrations in those patients were (usually well) above 0.1 µg/ml. We tested different adalimumab cut-off concentrations of 1.1, 3.1 and 5.1 µg/ml, but the results from all analyses were comparable (fig. S8). Also at week 52 and during steady state TNF concentrations were significantly lower in patients with detectable ADAs (p=0.0001 and p<0.0001; figs. S6C and S6D, respectively).

Finally, we observed that patients concomitantly treated with methotrexate had significantly higher median TNF concentrations at week four (11 (5.0-37)) compared to patients treated without methotrexate (173 (78-305) pg/ml, p<0.001; fig. S9).

TNF concentrations and ADAs in healthy volunteers

As we demonstrated that TNF remained stable during long-term follow-up, irrespective of disease activity, we asked whether circulating TNF could also be observed in similar quantities in healthy individuals. Therefore, we investigated TNF concentrations in healthy volunteers who had received a single dose of an adalimumab biosimilar, after which serum samples were frequently drawn. Similar to patients with RA, we found that baseline TNF concentrations were low in healthy volunteers, but TNF rapidly increased within half a day after adalimumab administration (Fig. 6A). After a peak concentration at day seven, TNF concentrations differentially started to decrease in a subset of subjects (n=12). This decrease in TNF was associated with ADA formation (Fig. 6A), and an accelerated adalimumab clearance (fig. S10A). Strikingly, in 10 out of 12 (83%) ADA positive individuals, we observed a rapid drop in TNF at a time where adalimumab concentrations were still high and ADAs could not yet be detected (representative graph shown in Fig. 6B, additional examples in fig. S10C). In other words, the decrease in TNF preceded the accelerated clearance of adalimumab, which in turn preceded the detection of ADAs.

Discussion

Little is known about circulating TNF during TNF-inhibitor treatment due to technical difficulties in quantifying TNF bound to the TNF-inhibitor. To investigate the relationship between TNF concentrations and clinical response during TNF-inhibitor treatment, we developed a drug-tolerant competition ELISA. This assay quantified total TNF concentrations during adalimumab treatment, which mainly comprises inactive, drug-bound TNF. We demonstrated that after an initial and often steep (>50-fold) rise, TNF concentrations stabilized and remained stable during two years of follow-up and did not drop in patients with RA in remission. An increase in TNF concentrations upon starting anti-TNF treatment has been reported previously, albeit at much lower quantities *(1–5, 24)*, most likely due to interference of the TNF-inhibitor with the quantification of TNF in conventional TNF ELISAs *(3)*. Furthermore, TNF concentrations also increased upon adalimumab biosimilar administration in healthy volunteers, reaching concentrations comparable to those in patients with RA. Together, these findings indicate that TNF concentrations in circulation do not reflect (suppressed) inflammation, and that the majority of TNF likely does not originate from pathological processes. Instead, we showed a

strong association between early low TNF concentrations and future ADA formation in healthy volunteers and in patients with RA. Early (week 4) low TNF concentrations in patients with RA were associated with less frequent remission after 52 weeks. Early TNF concentrations therefore may be developed as a biomarker to predict future ADA formation and to identify non-responders in the early phase of treatment.

We demonstrated that patients who were treated without concomitant methotrexate had lower TNF concentrations. As known from previous studies, methotrexate is inversely related with the detection of ADAs *(18, 19)*. The relationship between low TNF concentrations and ADA detection against a background of adalimumab concentrations >0.1 µg/ml persisted also for prolonged periods of time; TNF concentrations at week 52 and at steady state were significantly lower in those patients with detectable ADAs.

Of note, ADAs were measured with a drug-sensitive antigen binding test (ABT), which has been shown to correlate with clinical efficacy *(20)*. In contrast, a drug-tolerant acid-dissociation radioimmunoassay (ARIA) is of limited predictive value for clinically relevant ADA formation, partially due to antibody responses being transient in a subset of patients *(21, 22)*.

The relation between early low TNF and ADA formation was even more pronounced in healthy volunteers who had received one dose of adalimumab biosimilar. TNF concentrations rapidly decreased in a subset of individuals, before non-linear, ADA-associated clearance of adalimumab was observed. In other words, at the moment that TNF concentrations started to decline, the adalimumab concentration was still sufficiently high for quantitative capture of TNF. This decline in circulating TNF might be associated with decreased TNF production on the one hand, or enhanced TNF clearance on the other hand. As TNF is an important mediator driving immune responses *(23)*, reduced TNF production linked to antibody formation does not seem very likely. Alternatively, we hypothesize that during the early phase of the anti-adalimumab immune response, low-affinity, adalimumab-specific B-

cells and/or ADAs (possibly IgM) are present that might preferentially bind 3:1 adalimumab:TNF complexes over unbound single adalimumab molecules. This could result in selective uptake and/or clearance of the adalimumab:TNF complexes by macrophages at this stage, translating to a severe drop in TNF concentrations, while there is not yet a measurable effect on adalimumab serum concentration. In time, affinity-matured (IgG) ADAs develop, coinciding with increased clearance of adalimumab and the detection of ADAs.

The strong association between ADA formation over 52 weeks and low TNF concentrations at week four in patients with detectable adalimumab concentrations (>0.1 µg/ml) is different from the observation that TNF concentrations significantly dropped due to ADA formation in some patients. The latter ADA formation led to undetectable amounts of adalimumab, and, consequently, the disappearance of TNF. For quantitative capture of TNF, which results in the detection of TNF-adalimumab complexes, a minimal critical amount of adalimumab is thus required. Our data suggest that an adalimumab concentration around as low as 0.1 µg/ml is sufficient for near-quantitative *in vivo* capture of TNF. Above this adalimumab concentration, the vast majority of TNF will be in complex with the drug and the concentration of TNF does not appreciably depend on serum drug concentration. This was further supported by data from patients who discontinued adalimumab treatment, in which we found that TNF concentrations remained stable in patients with an adalimumab concentration above 0.1 µg/ml (even 24 weeks after treatment discontinuation). One may wonder at which point in time a patient can truly be classified as having discontinued adalimumab treatment. Furthermore, this observation may also have implications for patients who are advised to discontinue anti-TNF treatment in case of an infection or surgery, and for the evaluation of the success rate of treatment discontinuation studies. Of note, the number of patients was too small to investigate the relationship between TNF concentrations and clinical outcome, i.e. having a flare after dose-interval prolongation or treatment discontinuation. One should also keep in mind that although peripheral concentrations

of drug and target are supposed to be a good surrogate, concentrations at the site of inflammation might differ.

Adalimumab-TNF complexes were most likely formed in a 3:1 ratio, in line with several previous studies *(25, 26)*. Other studies, however, observed a variety of complexes with a wide range in size and stoichiometry for adalimumab and infliximab, but not for etanercept *(27–29)*. It was suggested that these differences in complex size may account for the difference in efficacy and side-effects of the different TNF-inhibitors. However, these studies made *in vitro* complexes using high concentrations of TNF and near-equimolar amounts of TNF-inhibitors. These concentrations do not reflect the *in vivo* situation, where a large excess of adalimumab over TNF in patients with RA is present. Our data show that the complex formation of TNF with adalimumab prolongs the TNF half-life, similar as has been shown for IL-6 *(11)*. However, in case of IL-6, complexes will contain only one molecule of anti-IL-6 (bound to either one or two molecules of IL-6), and it is expected that IL-6-anti-IL-6 complexes have a half-life approaching the free antibody. We do not know the impact of additional Fc domains in the adalimumab-TNF complexes on clearance rate. Fcγ-receptor-mediated uptake may vary substantially with the number of Fc domains in an immune complex *(30, 31)*, and so we cannot estimate the half-life of TNF-adalimumab complexes in circulation.

There are some limitations to this study. Cohort samples were prospectively collected, whereas retrospective analyses have been performed. Furthermore, although our results indicate that an adalimumab concentration of ca. 0.1 µg/ml is sufficient for quantitative capture of TNF, the number of data points in the critical window just above and below 0.1 µg/ml is limited, which impairs the precision of this estimate. A treatment discontinuation study with longer follow-up would allow closer monitoring of serum samples with adalimumab concentrations within this critical window.

Overall, these findings indicate that TNF cannot be used as a biomarker for treatment discontinuation. However, low early TNF concentrations can be used as an indicator to predict future ADA formation in the early phase of treatment.

Materials and Methods

Study design

This study was designed to explore the dynamics of circulating TNF during adalimumab treatment. First, a drug-tolerant TNF ELISA was developed, which allowed the quantification of TNF in the presence of a vast excess of TNF-inhibitor. This assay was used to quantify TNF in three groups of adalimumab-treated patients with RA: (i) during standard-dose treatment in a large prospective observational cohort, (ii) after dose-interval prolongation in a randomized, open-label, non-inferiority trial and (iii) after treatment discontinuation. The study groups were prospectively collected and retrospective analyses have been performed. Quantification of TNF was performed in a blinded fashion. For the analysis between week four TNF concentrations and ADA formation at week 52, only patients with an adalimumab concentration >0.1 µg/ml were included. Numbers of patients in the

analyses are specified in each figure. The finding that early low TNF concentrations associate with future ADA formation and potentially could serve as timely predictor of non-response, was validated in a second independent cohort. The association between TNF concentrations and future ADA formation was also validated in healthy volunteers administered an adalimumab biosimilar. Primary data are reported in table S2.

Assay development

Production of a recombinant high-affinity adalimumab mutant

First, a high-affinity adalimumab mutant (variant cb1-3) *(16)* was produced. Synthetic DNA constructs for variable light, constant light (VLCL) and variable heavy (VH) chains were ordered (Life Technologies) and cloned into pcDNA3.1 (Invitrogen) expression vectors, together with the constant domain of human IgG1, as described previously *(32)*. These expression vectors allowed transient transfection of HEK293F cells with 293fectin and OptiMEM (Invitrogen), using the Freestyle HEK293F expression system (Invitrogen) according to the manufacturer's instructions.

Analysis of antigen binding

A TNF inhibition ELISA was used to assess the binding of adalimumab (Humira) and the high-affinity adalimumab mutant to TNF, as described by van de Bovenkamp *et al*. *(33)*. In short, microtiter plates were coated with monoclonal mouse anti-TNF clone 7 and incubated with recombinant TNFα. Next, a titration of adalimumab or the high-affinity adalimumab mutant was added. Rituximab (Mabthera) was used as a negative control. Biotinylated adalimumab was added without washing plates. Finally, plates were incubated with streptavidin-polymerized horse radish peroxidase (poly-HRP) and developed with tetramethylbenzidine (TMB) substrate.

Drug-tolerant TNF competition ELISA

Nunc MaxiSorp 96-well flat bottomed plates (Thermo Scientific) were coated overnight at room temperature with 100 µl/well of 3 µg/ml monoclonal mouse anti-human TNF (clone 7, Sanquin Reagents) in phosphate buffered saline (PBS). After washing five times with PBS containing 0.02% Tween (PBS-T), samples, diluted five-fold in high performance ELISA (HPE) buffer (Sanquin Reagents) supplemented with 1 mg/ml IVIg, to minimize non-specific binding, (Nanogam, Sanquin) (HPE+) were incubated for one hour at room temperature on a shaker platform. Plates were washed five times with PBS-T and 100 µl of 0.5 µg/ml biotinylated high-affinity adalimumab mutant antibody in HPE+ buffer was added for detection. After two hours at 37°C on a shaker platform, plates were washed five times with PBS-T and incubated with 100 µl of streptavidin poly-HRP (1:10000 dilution in HPE buffer) for 25 minutes at room temperature on a shaker platform. After five times washing with PBS-T, 100 µl of TMB substrate (100 µg/ml) and 0.003% (v/v) hydrogen peroxide (Merck) in 0.11 M sodium acetate buffer (pH 5.5) was added to each well. The reaction was stopped by adding 100 µl of 2M H_2SO_4 (Merck) and optical density (OD) was measured at 450 nm and 540 nm with a plate reader (Synergy 2, Bio Tek). TNF concentrations were calculated with a serially 2-fold diluted calibration curve of TNF in HPE+ buffer, that was calibrated against the WHO standard. A cut-off was determined as the mean + 3 SD of healthy donor sera (n=70). Lack of substantial interference by rheumatoid factor (RF) was inferred from absence of significantly higher signals in RF positive, biologic naïve RA patient sera vs RF negative, biologic naïve RA patient sera.

TNF-adalimumab complex ELISA

A complex ELISA was developed in order to specifically quantify TNF-adalimumab complexes (fig. S3A). Monoclonal mouse anti-human TNF (clone 7, Sanquin Reagents) was diluted to 2 µg/ml in PBS and used for coating Nunc MaxiSorp 96-well flat bottomed plates (Thermo Scientific) with 100 µl/well overnight at room temperature. After washing five times with PBS-T, samples were five-fold diluted in HPE+ buffer and incubated for one hour at room temperature on a shaker platform. Plates were washed five times with PBS-T, followed by incubation with 100 µl/well of biotinylated polyclonal

adalimumab specific rabbit-anti-idiotype antibody (0.125 µg/ml in HPE buffer) for two hours at room temperature. Plates were washed five times with PBS-T. Then 100 µl of streptavidin poly-HRP (1:10000 dilution in HPE buffer) was added to each well. After 25 minutes, plates were washed five times with PBS-T and incubated with 100 µl of TMB substrate (100 µg/ml) and 0.003% (v/v) hydrogen peroxide (Merck) in 0.11 M sodium acetate buffer (pH 5.5). 100 µl/well of 2M H_2SO_4 (Merck) was used to stop the reaction. Absorbance was measured at 450 nm and 540 nm with a plate reader (Synergy 2, Bio Tek). Concentration of TNF-adalimumab complexes was determined with a serially 2-fold diluted calibration curve of TNF (Active Bioscience) in HPE+ buffer, which was incubated 1:1 with 10 µg/ml adalimumab (5 µg/ml final concentration in the assay) for 30 minutes at room temperature, before adding 100 µl/well.

A selection of serum samples (n=36) sent in to Sanquin Diagnostic Services were measured in parallel in both the competition and complex ELISA. These samples were leftovers from samples taken for routine diagnostic purposes. No ethics approval was obtained, but patients had approved that samples could be used for research purposes. Materials were used anonymously without any connection to clinical data. The adalimumab concentration and ADA titer of these samples had been determined previously at Sanquin Diagnostic Services.

Characterization of TNF-adalimumab complexes

High-performance size-exclusion chromatography (HP-SEC)

Selected serum samples from patients treated with standard-dose adalimumab were fractionated by HP-SEC to characterize TNF-adalimumab complexes *ex vivo*. Serum was diluted 1:1 in PBS and filtered (0.22 µm filter, Merck Millipore) before applying 500 µl to a Superdex 200 GL 10/300 column (GE Healthcare) and eluted with PBS at 0.5 ml/min. Elution profiles of complexes were monitored by measuring absorption at 280 nm with an ÄKTA explorer HPLC system (GE Healthcare). Samples spiked with free TNF (Active bioscience, stored in 6% human serum albumin (HSA)) (500 pg/ml TNF in PBS, supplemented with 5 mg/ml IVIg) and TNF-adalimumab complexes (500 pg/ml TNF and 5 µg/ml

adalimumab in PBS, supplemented with 5 mg/ml IVIg) were used as controls. Additionally, TNF-adalimumab complexes with different ratios of TNF:adalimumab were fractionated (500 pg/ml TNF and 5, 1.5 or 0.5 µg/ml adalimumab in PBS, supplemented with 5 mg/ml IVIg). Fractions of 250 µl were collected in 27 µl HPE buffer (5x concentrate), supplemented with 10 mg/ml IVIg and stored at -20°C until TNF concentrations were measured in (undiluted) fractions with the drug-tolerant TNF competition ELISA, as described above.

WEHI bioassay

TNF bioactivity in serum samples of patients with RA during standard-dose adalimumab treatment was determined with a TNF-sensitive WEHI bioassay. 70 serum samples with measurable TNF during adalimumab treatment were randomly selected. Nunc MicroWell Plates with Nunclon Delta Surface (Thermo Scientific) were plated with 40,000 WEHI-164 cells (ATCC CRL-1751)/well in 50 µl IMDM (Bio Whittaker) supplemented with 5% fetal calf serum (FCS) (Bodinco), 100 U/ml penicillin, 100 µg/ml streptomycin (both from Gibco), 1 µg/ml Actinomycin D and 50 µM β-Mercapto-ethanol (both from Sigma) (assay medium). Subsequently, cells were either incubated 1:1 with a titration of TNF (Active Bioscience) in a final concentration of 0 – 10,000 pg/ml or with 1:20 diluted patient serum (diluted in assay medium). A condition without TNF (non-inhibited sample) was included as reference. After 24 hours, cell viability was determined with the MTT-reduction method. MTT (Sigma, diluted in 0.14 M NaCl and 0.01 M HEPES) was added in a final concentration of 0.83 mg/ml. After four hours, 5% SDS (Gibco, diluted in 0.01 M HCL) was added and incubated overnight. OD was measured at 595 nm and 670 nm with a plate reader (Synergy 2, Bio Tek). All incubation steps were performed at 37°C and 5% CO_2. All conditions were analyzed in triplicate.

Measurement of adalimumab and anti-adalimumab concentrations

Trough adalimumab concentrations were measured by ELISA, as previously described *(21, 34)*. In short, plates coated with mouse monoclonal anti-human TNF clone 7 were incubated with recombinant

TNFα. Next, patient sera was added and incubated with biotinylated polyclonal adalimumab specific rabbit-anti-idiotype antibody for detection. Adalimumab concentrations were calculated with an adalimumab titration curve. The lower limit of quantification (LLOQ) was 0.01 µg/ml.

The antigen binding test (ABT), as reported previously *(35)*, was used to measure anti-adalimumab antibodies. Antibodies present in serum were captured overnight by protein A Sepharose. Adalimumab-specific antibodies were detected with ^{125}I (Perkin Elmer)-labeled F(ab)2 adalimumab. Unbound label was removed by washing five times and Sepharose-bound radioactivity was measured. Antibody concentrations were calculated with a reference serum and expressed in arbitrary units (AU/ml). Limit of detection (LOD) was 12 AU/ml.

Patients

Three groups of patients were studied, each with a different type of treatment regimen. Longitudinal TNF concentrations during standard-dose adalimumab treatment were studied in the first group, comprising 193 consecutive biologic naïve patients with RA from the Reade Rheumatology Registry, a prospective observational cohort study (Dutch Trial Register NTR no. 6868). A proportion of these patients was previously described *(20)*. Patients were treated with a standard-dose adalimumab of 40 mg subcutaneously every other week. At the start of adalimumab treatment patients had an active disease, i.e. disease activity score of 28 joints, including erythrocyte sedimentation rate (DAS28-ESR) >3.2, in agreement with the Dutch consensus statement on the initiation and continuation of TNF blocking therapy in RA. Patients were enrolled between February 2004 and December 2007. Serum samples were drawn at baseline (week 0) and prior to the next adalimumab injection at week 4, 16, 28, 40, 52, 78, and 104. To validate the primary clinical results, we included another 193 consecutive patients with RA from the Reade Rheumatology registry. These patients were included between October 2007 and January 2013.

To elucidate the relationship between (decreasing) adalimumab concentrations and the TNF concentration in circulation, two other study populations were included. One comprised consecutive patients with RA (n=21) from a randomized, open-label, non-inferiority trial (Dutch Trial Register NTR no. 3509). The patients were overexposed to drug, with an adalimumab concentration above 8 µg/ml, as previously described *(36)*. The patients successfully completing 28 weeks of follow up after dose-interval prolongation of 40 mg adalimumab every three weeks were included. In this study, serum samples were obtained prior to the intervention, and 12 and 28 weeks thereafter.

The final group comprised 11 consecutive patients with RA with low disease activity, successfully completing at least 12 weeks of follow-up after adalimumab treatment discontinuation in the Reade Rheumatology Registry between April 2012 and July 2013. Low disease activity was defined as DAS-ESR <3.2. Before treatment discontinuation, patients had been treated with adalimumab 40 mg every other week. Citrate plasma samples were obtained prior to treatment discontinuation and 12 and 24 weeks thereafter.

In all three study groups, patients were seen in the Amsterdam Rheumatology and immunology Center, Reade, Amsterdam, the Netherlands, and patients fulfilled the American college of Rheumatology 1987 revised criteria for RA *(37)*. Apart from adalimumab (Humira; AbbVie) most patients were treated with concomitant DMARDs, including methotrexate. All protocols were approved by the medical ethics committee of the Slotervaart Hospital and Reade Medical Research and all patients gave written informed consent.

Pfizer adalimumab biosimilar study in healthy volunteers

30 heathy volunteers received one dose of Pfizer proposed adalimumab biosimilar. Serum samples were drawn before, and frequently after adalimumab administration at day 0.5, 1, 3, 7, 14, 21, 28, and 42. Approval was obtained from Integreview IRB, 3815 S. Capital of Texas Hwy, Ste 320, Austin, TX 78704, United States.

Clinical outcomes

In the standard-dosed group clinical and laboratory assessments were at baseline and 4, 16, 28, 40, 52, 78, and 104 weeks thereafter and comprised: tender joint count, swollen joint count, patient's assessment of pain (visual analogue scales (VAS) 0-100 mm), patient's global assessment of disease activity (VAS 0-100 mm), physician's global assessment of disease activity (VAS 0-100 mm), ESR, C-reactive protein (CRP), current medication use and Health Assessment Questionnaire (HAQ). Variables additionally recorded at baseline were: age, gender, length, weight, duration of disease, IgM rheumatoid factor (RF) and anti-citrullinated peptide antibody (ACPA) status, medication history regarding prior and current DMARD therapy, glucocorticoid and TNF-inhibitor use.

Statistical analysis

In the standard-dosed group, the relationship between TNF concentration and SDAI remission at baseline and after 52 weeks was analyzed with a Spearman's rank correlation test. TNF concentrations were investigated at week four, week 52, and at steady state. This latter steady state TNF concentration was determined as the median concentration of three visits between 28 and 52 weeks (n=105). In case serum samples were only available at two out of three time points in this period, the mean concentration was calculated (N=36). No steady state could be determined in patients with only one visit (n=10). The steady state concentration and concentration at week 52 reflects a responder-analysis, since most non-responders will drop out before 28 weeks. In addition, the association between TNF at week four, week 52 or at steady state and also methotrexate use at baseline and ADA detection during 52 weeks follow-up were tested with a Mann-Whitney U test. For the analysis of TNF at week four, last observation carried forward was used for SDAI scores for those patients that discontinued adalimumab treatment prior to week 52. A ROC analysis was conducted to obtain a representative cut-off value for TNF concentrations at week four, to predict ADA detection after 52 weeks of adalimumab treatment. Additionally, methotrexate was included as a covariable in this analysis. In order to investigate the relationship between TNF and adalimumab concentrations, an

overall Spearman's correlation coefficient was determined. For all analyses SPSS for Windows version 21.0 or GraphPad Prism version 7.04 were used; ROC analysis was carried out using R v3.4.3 using the ROCR package. A P-value <0.05 (2-sided) was considered significant.

Supplementary Materials

Fig. S1. Analysis of antigen binding.

Fig. S2. TNF concentrations measured with the drug-tolerant competition ELISA

Fig. S3. Development of a TNF-adalimumab complex ELISA.

Fig. S4. Adalimumab:TNF complexes.

Fig. S5. Correlation between TNF and adalimumab concentrations.

Fig. S6. TNF concentrations at week 52 and at steady state in relation with clinical response.

Fig. S7. Validation of the relation between week four TNF concentrations and clinical response.

Fig. S8. Different adalimumab cut-off concentrations did not affect the relation between TNF at week four and clinical response.

Fig. S9. Week four TNF concentrations in relation with baseline methotrexate use.

Fig. S10. Adalimumab concentrations and anti-drug antibodies in healthy volunteers.

Table S1. Demographics of validation cohort.

Table S2. Primary data.

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Competing interests MTN reports having received consultancy fees from Abbott, Roche, Pfizer, MSD, UCB, SOBI and BMS, payment for lectures from Abbott, Roche and Pfizer. RFvV has received research grants from AbbVie, BMS, GSK, Pfizer and UCB and consultancy honoraria from AbbVie, AstraZeneca, Biotest, BMS, Celgene, GSK, Janssen, Lilly, Novartis, Pfizer and UCB. MB has received consultancy fees from BMS, UCB and Teva. DFA is an employee of Pfizer Inc. CHS reports departmental funding from AbbVie, Janssen, Leo and Pfizer. GJW has received a research grant from Pfizer (paid to the institution) and honoraria for lectures from Pfizer, UCB, AbbVie, Biogen and BMS. TR has received honoraria for lectures from Pfizer, AbbVie and Regeneron, and a research grant from Genmab.

Data and materials availability: All data associated with this study are present in the paper or Supplementary Materials.

Figures

Fig. 1. Development of a drug-tolerant competition ELISA. (**A**) Schematic overview of the drug-tolerant competition ELISA. Both free TNF and TNF-adalimumab complexes in serum are bound to an anti-TNF coating antibody. An excess of a biotinylated high-affinity adalimumab mutant antibody is added, which will result in the displacement of adalimumab from TNF, allowing efficient detection of TNF. Measurement of free TNF and TNF-adalimumab complexes in the drug-tolerant competition ELISA (**B**) and in a conventional TNF ELISA (**C**). Shown is a representative titration of free TNF, preincubated in absence or presence of 5 µg/ml adalimumab of at least 4 independent experiments.

Fig. 2. Characterization of TNF-adalimumab complexes. (**A**) Correlation between TNF measured in sera, sent in to Sanquin Diagnostic Services, using the competition ELISA and complex ELISA (Pearson $r=0.96$, $p<0.0001$ ($n=36$)). Samples spiked with free TNF (**B**) and TNF preincubated with adalimumab (**C**) in buffer containing IVIg and albumin were fractionated with HP-SEC (gray line). The two A280 peaks around 12.5 and 14 ml represent IgG and albumin, respectively. TNF was measured in collected fractions using the drug-tolerant competition ELISA (black line). Representatives of at least two independent experiments are shown. (**D**) Characterization of TNF-adalimumab complexes in serum derived from adalimumab-treated patients ($n=4$). A representative example of a patient is shown.

Fig. 3. Quantification of TNF in adalimumab-treated patients with RA. (**A**) TNF serum concentrations were determined at baseline (week 0) and after 4, 16, 28, 40, 52, 78, and 104 weeks of adalimumab treatment in 193 patients with RA, using the drug-tolerant competition ELISA. Each dot represents mean TNF concentration of a duplicate measurement in an individual patient; black lines show median

(IQR). (**B**) TNF activity in a selection of RA patient sera (n=70) during adalimumab treatment. WEHI-164 cells were incubated with 1:20 diluted patient serum (diluted in assay medium), in triplicate. Dashed line shows the OD of a non-inhibited reference sample. (**C**) Representative examples of patients with increased TNF concentrations during the first phase of treatment and stabilized TNF concentrations over time. (**D**) Representative examples of patients with diminished TNF concentrations over time. (A, C-D) Dashed lines represent cut-off of 5 pg/ml TNF.

Fig. 4. Quantification of TNF in patients with RA after adalimumab treatment prolongation or treatment discontinuation. (**A**) Longitudinal adalimumab concentration or (**B**) TNF concentration in 21 patients with RA, before (week 0) and 12 and 28 weeks after treatment prolongation. (**C**) Longitudinal adalimumab serum concentration or (**D**) TNF concentration in 11 patients with RA, before (week 0) and 12 and 24 weeks after adalimumab treatment discontinuation. Colored lines in C correspond to patients with similar colored lines in D. (B,D) Dashed lines indicate cut-off of 5 pg/ml TNF. (C) Dashed line indicates LLOQ of 0.01 µg/ml adalimumab.

Fig. 5. Week four TNF concentrations in relation with clinical response. (**A**) Correlation between TNF concentrations at week four and disease activity, according to SDAI, at week 52. (Spearman's ρ=-0.21, P<0.005 (n=168)). Gray line indicates log-log linear fit, weight by 1/Y^2. (**B**) TNF concentrations were stratified by ADA detection during 52 weeks of follow-up. Each dot represents mean TNF concentration of a duplicate measurement in an individual patient; black lines show median (IQR); patients were only included in the analysis if (free) adalimumab concentrations exceeded 0.1 µg/ml. ****p<0.0001, Mann-Whitney U test. (A-B) Dashed lines indicate cut-off of 5 pg/ml TNF. (**C**) ROC analysis of TNF concentrations at week four (black line). In order to predict ADA formation versus no ADA formation after 52 weeks of adalimumab treatment an AUC of 0.79 was found, and a cut-off value of 11 pg/ml TNF yielded 51% sensitivity and 95% specificity. Inclusion of baseline methotrexate usage in the model resulted in an AUC of 0.81 (gray line).

Fig. 6. Quantification of TNF in healthy volunteers. (**A**) TNF serum concentrations were determined before (day 0), and frequently after one dose of an adalimumab biosimilar in 30 healthy volunteers, using the drug-tolerant competition ELISA. TNF concentrations were stratified by volunteers in whom no ADAs were detected (gray triangles (n=18)) and volunteers with detectable ADAs over 42 days of follow-up (orange dots (n=12). Each symbol represents mean TNF concentration of a duplicate measurement in an individual volunteer; gray and orange lines show median TNF for ADA negative and ADA positive, respectively. (**B**) The dynamics in TNF (black dots), ADA titer (gray triangles; both left y-axis) and adalimumab concentration (orange squares; right y-axis) in a volunteer who became ADA positive. A representative example of one healthy volunteer is shown. Black and gray dashed lines indicate a cut-off of 5 pg/ml TNF or an LOD of 12 AU/ml anti-adalimumab antibodies, respectively.

Table

Table 1. Demographics, previous and concomitant therapies, and disease status at baseline

	Patients (n=193)
Demographics	
Age, mean ± SD years	54 ± 11
Female, No. (%)	148 (77)
BMI, mean ± SD	25.7 ± 5.1
DMARD therapy	
Prior DMARDs, median (IQR)	3 (2-4)
MTX use, No. (%)	149 (77)
MTX dose, median (IQR) mg/week	25 (15-25)
Prednisone use, No. (%)	60 (31)
Prednisone dose, median (IQR), mg/day	7 (5-10)
Disease Status	
Disease duration, median (IQR) years	8 (3-17)
ACPA positive, No. (%)	138 (72)
IgM-RF positive, No. (%)	135 (70)
Erosive, No. (%)	143 (74)
DAS28, mean ± SD	5.0 ± 1.3
SDAI, mean ± SD	21 (14-27)
ESR, median (IQR) mm/hour	21 (9-42)
CRP, median (IQR) mg/L	11 (4-23)
HAQ, median (IQR)	1.1 (0.8-1.6)

SD = standard deviation; No = Number; IQR = inter quartile range; BMI = body mass index; DMARD = disease-modifying antirheumatic drug; MTX = methotrexate; ACPA = anti-citrullinated protein antibody; IgM-RF = IgM rheumatoid factor; DAS28 = 28-joints disease activity score; SDAI = simplified disease activity score; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; HAQ = Health Assessment Questionnaire.